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# *Snail2* and *Zeb2* repress *P-Cadherin* to define embryonic territories in the chick embryo

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**Summary statement:** The chick embryo uses P-cadherin as the main epithelial cadherin at early stages. Sequential P-Cadherin repression in the primitive streak and the neural plate defines embryonic territories.

**KEY WORDS:** E-Cadherin repressors, EMT, P-Cadherin, Sip1, gastrulation, neural plate

**ABSTRACT**

**Snail and Zeb transcription factors induce epithelial to mesenchymal transition (EMT) in embryonic and adult tissues by direct repression of *E-Cadherin* transcription. The repression of E-Cadherin transcription by the EMT inducers Snail1 and Zeb2 plays a fundamental role in defining embryonic territories in the mouse, as E-Cadherin needs to be downregulated in the primitive streak and in the epiblast concomitant with the formation of mesendodermal precursors and the neural plate, respectively. Here we show that in the chick embryo, *E-Cadherin* is weakly expressed in the epiblast at pre-primitive streak stages where it is substituted by *P-Cadherin*. We also show that *Snail2* and *Zeb2* repress *P-Cadherin* transcription in the primitive streak and the neural plate, respectively. This indicates that *E-* and *P-Cadherin* expression patterns evolved differently between chick and mouse. As such, the Snail1/E-Cadherin axis described in the early mouse embryo corresponds to Snail2/P-Cadherin in the chick, but both Snail factors and Zeb2 fulfill a similar role in chick and mouse in directly repressing ectodermal *Cadherins* to promote the delamination of mesendodermal precursors at gastrulation and the proper specification of the neural ectoderm during neural induction.**

## INTRODUCTION

During early embryonic development, the embryo progresses from a single layer of epithelial cells (the epiblast) to a three-dimensional structure composed of several layers and territories. As part of this complex process, embryonic cells integrate environmental cues to acquire positional information, fate specification and control of cell behaviours, resulting in the formation of embryonic layers, either by modelling epithelial sheets or by inducing individual or collective cell migration. The Epithelial to Mesenchymal Transition (EMT) enables delamination at the primitive streak to give rise to the definitive endoderm and mesoderm while cells that remain in the epiblast at gastrulation contribute to the ectoderm (reviewed by Acloque et al., 2009).

The EMT program is triggered by the activation of transcription factors called EMT-TFs that include the *Snail* and *Zeb* families (i.e. Nieto et al., 1994, Vandewalle et al. 2009), which directly repress *E-Cadherin* transcription, confirming their crucial role in modulating cell adhesion (Batlle et al., 2000; Cano et al., 2000, Eger et al. 2005, Comijn et al. 2001). *Snail1* in mammals and *Snail2* in birds are expressed in the ingressing cells at the primitive streak, in neural crest cells delaminating from the neural tube, in the presomitic mesoderm and the lateral plate mesoderm among other EMT territories (Nieto et al. 1994, Acloque et al 2011, Blanco et al. 2007, Morales et al. 2007, Dale et al. 2007, Niessen et al. 2008). *Snail1* mouse mutants maintain high levels of E-cadherin at the primitive streak, do not complete EMT and the resulting defective mesoderm fails to migrate (Carver et al. 2001). In chick and mouse, *Zeb2* (also known as *SIP1*) is expressed in the neural plate and neural tube. Zeb2 does not induce EMT, as these territories remain epithelial all throughout neurulation, but defines the neural versus the non-neural ectoderm (Van de Putte 2003, Van Grunsven et al. 2007 Vandewalle et al. 2009). Like *Snail* mutants, *Zeb2* mutant embryos maintain E-cadherin expression in the corresponding territories, the neural plate and in the presumptive neural crest. These mice exhibit multiple neural crest defects, fail to specify the neuroepithelium correctly and die right after neurulation, at E9.5 (Van de Putte 2003). All these data support the importance of E-cadherin repression in the definition of embryonic territories and subsequent tissue differentiation in the mouse. In the chick embryo, L-CAM was proposed to be the functional equivalent of E-cadherin in the mouse because it is expressed in the chick epiblast (Dady et al. 2012, Ohta et al. 2007). However, *L-CAM* is only faintly expressed in the epiblast of pre-primitive-and primitive streak chick embryos (Moly et al. 2016 and this work). The chicken genome includes another type I cadherin, located in a cluster

adjacent to *L-CAM* (E-Cadherin). Because this is reminiscent of the organization of *P-* and *E-Cadherin* in mammals, Redies and Müller (1994) proposed that this is the homologue of *P-Cadherin* in the chick. Here we show that the chick embryo mostly expresses *P-Cadherin* instead of *E-Cadherin* in the epiblast and that, like E-Cadherin in the mouse, P-Cadherin is downregulated in the mesoderm and in the induced neural plate while it is maintained in non-neural ectoderm. Since *P-Cadherin* expression is complementary to that of *Snail* and *Zeb* genes, we performed gain and loss of function experiments to evaluate whether these epithelial repressors are responsible for the downregulation of *P-Cadherin* during primitive streak stages, when *Snail2* is expressed in the streak and in the ingressing mesendoderm and at neurulation stages, when *Zeb2/Sip1* is expressed in the early neural plate. We find that Snail2 and Zeb2 repress *P-cadherin* expression in the chick embryo as Snail1 and Zeb2 repress *E-cadherin* in the mouse, indicating a reshuffling in the expression of Snail and Cadherin family members and a functional conservation in the mechanism that helps define embryonic territories in vertebrates.

## RESULTS AND DISCUSSION

### Expression profiles of type I Cadherins differ between chick and mouse embryos

It is currently assumed that as in mammals, E-Cadherin (L-CAM) is expressed in the epiblast of the chicken embryo (Bobbs et al. 2012, Thiery et al. 1984). However, *in situ* hybridization for *E-Cadherin* reveals very weak expression before Hamburger and Hamilton stage (HH) 9 (Hamburger and Hamilton, 1951) (Fig. 1A, D; G, Moly et al. 2016). To assess whether another cadherin could substitute for *E-Cadherin* in the early chick embryo, we examined the expression of other type I cadherins and observed *N-Cadherin* strongly expressed in the early mesoderm as previously described (Fig. 1B; Hatta and Takeichi 1986, Garcia-Castro et al. 2000), but also strong expression of *P-Cadherin* in the epiblast (Fig. 1C and F). Real time RT-PCR on embryos at pre-primitive streak (EGXI-XIII) (pre-primitive streak stages according to Eyal-Giladi and Kochav, 1976; EG), primitive streak (HH4) and neurulation (HH9) stages and in chick embryonic fibroblasts (CEFs) shows that *P-Cadherin* is expressed around 10-times more strongly than *E-Cadherin* at EGXI and HH4 and 2-times more at HH9 and *N-Cadherin* is predominantly expressed in CEFs and to a lesser extent at HH4 and HH9 (Fig. S1), all

confirmed by *in situ* hybridization at equivalent stages (Fig. 1A-I). Thus, *P-Cadherin* is the predominant type-I cadherin expressed in the chick embryo epiblast at pre-primitive and primitive streak stages. Double *in situ* hybridization for *P-* and *N-Cadherin* in gastrulating embryos (Fig. 1J) shows patterns reminiscent of those described in the mouse for *E-* and *N-Cadherin,* respectively (Radice et al. 1997). In the mouse, *P-cadherin* is not expressed in the epiblast and appears in the extraembryonic ectoderm and in the visceral endoderm, and later in various embryonic epithelia (Nose and Takeichi 1986, Hirai et al. 1989, Palacios et al. 1995, Lin and DePhilip 1996, Xu et al. 2002). Our data suggest an exchange between the expression of *P-* and *E-cadherin* in chicken and mouse, reminiscent of the swap that occurred between the two transcription factors, *Snail1* and *Snail2,* during evolution (Locascio et al. 2002). *Snail2* in the chick shows a pattern of expression reminiscent of that of *Snail1* in the mouse.

**Snail2 represses *P-Cadherin* in the epiblast of primitive streak stage chick embryos**

As expected because of its role as an *E-cadherin* repressor, the pattern of *Snail1* expression is complementary to that of *E-Cadherin* in the mouse embryo (Cano et al., 2000). Since in early chick embryos *P-Cadherin* is mostly expressed like mouse *E-Cadherin*, we examined whether *Snail2* and *P-Cadherin* are expressed in complementary patterns during gastrulation (Fig. 1K and L). *P-Cadherin* is mostly expressed in the ectoderm and its expression decreases in *Snail2* positive cells at the primitive streak and after ingression (Fig. 1M), compatible with the idea that Snail2 may be a repressor of *P-Cadherin*. To test this, we overexpressed Snail2 in the anterior epiblast of stage HH3 embryos. Embryos showed a decrease of *P-Cadherin* expression in the electroporated area as compared to the control side (Fig. 2 D-F, n=14/14) or with embryos electroporated with a GFP-only control construct (Fig. 2 A-C, n=11/11). Conversely, knock-down of *Snail2* using double-stranded RNA (dsRNA) (Pekarik et al. 2003) expands the territory of *P-Cadherin* expression up to the midline of the embryo at the primitive streak, where it is normally downregulated (Fig. 2G-I, n=4/6; see Fig. S2 to assess Snail2 downregulation, n=3). These data indicate that Snail2 represses *P-Cadherin* transcription in the chick epiblast.

We previously showed that overexpression of Snail2 in the chick epiblast induces ectopic EMT along with downregulation of Cadherin protein and disruption of the basement membrane, and that a similar mechanism operates in the mouse for Snail1 (Acloque et al 2011). While a recent study proposed that downregulation of P- and E-

Cadherin are not necessary for EMT to occur in the chick (Moly et al. 2016), our data, in addition to previous studies in various models (Ramkumar et al. 2016, Schäfer et al. 2014, Rogers et al. 2013, Carver et al. 2001, Wu and McClay 2007, Oda et al. 1998), confirm the downregulation of *E-* and *P-Cadherin* transcripts at sites of EMT and support a model in which their transcriptional downregulation is necessary for the transition towards a mesenchymal tissue arrangement. Since the half-life of E-Cadherin and β-catenin proteins at adherens junction can exceed 25h in epithelial cell lines (Lozano and Cano 1998) additional mechanisms favoring E-cadherin endocytosis and players such as Rho modulators, Crumbs2 or the MAP kinase pathway are fundamental to speed the turn-over and removal of E-cadherin in remodeled embryonic epithelia (Nakaya et al., 2008, Ramkumar et al. 2016, Moly et al., 2016; Zohn et al., 2006).

**Snail2 binds to *P-Cadherin* promoter *in vivo***

Snail factors repress *E-Cadherin* transcription directly by binding to specific E-boxes located in the *E-Cadherin* promoter (Cano et al. 2000; Batlle et al., 2000). We therefore examined whether Snail2 binds to the *P-Cadherin* promoter. As the sequence of P-Cadherin is incomplete at the 5' end of the gene in the public databases, we used RNA-seq data together with 5'-RACE and ESTs alignments to define a putative Transcription Start Site (TSS) of the *P-Cadherin* gene. After combining 5'-RACE and genomic DNA PCR amplification to get additional sequences, we aligned RNA-seq data on this reconstructed *P-Cadherin* locus and confirm the genomic structure of the *P-Cadherin* gene as containing 15 exons over 5.7kb (Fig. S3), encompassing the full coding region (2466bp) including the signal peptide (Brasch et al. 2012, Figure S2B). Our study completes the sequence of the chick *P-cadherin* gene from earlier studies (Sorkin et al. 1991, Napolitano et al. 1991) and supports the idea that *P-Cadherin* and *E-Cadherin* have undergone tandem duplication in birds and mammals.

Snail factors bind the consensus sequence CASSTG, which is over-represented (see RNA-seq analysis in M&M) in two regions upstream of the *P-Cadherin* promoter at positions between -1400bp and -2200bp from the TSS (Fig. 2J). To test whether Snail2 can bind to these response elements in epiblast cells, we electroporated GFP together with either a myc-tagged Snail2 or a myc-tagged control construct in the anterior epiblast of stage HH3 chick embryos. Ten hours after electroporation, GFP positive epiblast regions were dissected and processed for ChIP (Fig. 2K). Myc-tagged Snail2 overexpression led to a specific enrichment of regions B and C of the *P-Cadherin*

promoter after ChIP as assessed by precipitation with an anti-myc antibody followed by PCR with specific primers (Fig. 2 L-M). Positive (panH3 antibody) and negative (rabbit anti-IgG antibody) controls confirmed the specificity of the myc-antibody. The absence of enrichment for a control region (D) that does not contain Snail response elements confirms the specificity of Snail2 binding to regions B and C. Together, these results indicate that Snail can directly bind to and repress transcription of the *P-Cadherin* gene in vivo.

**P-Cadherin expression is downregulated in the neural plate concomitantly with activation of Zeb2**

After gastrulation, *P-Cadherin* expression decreases in the developing neural plate but remains expressed at its border (Fig. 3A). To identify putative transcription factors involved in this repression, we looked at EMT-related TFs expressed at neurulation stages in the chick embryo (Fig. 3B-E) and confirmed that *Zeb2* expression is expressed in the very early neural plate (Fig. 3E and Sheng et al., 2003), concomitant with the decrease of *P-Cadherin* expression. Double *in situ* hybridization comparing *Zeb2*, *P-Cadherin* and *Sox2* (a marker of the neural ectoderm), shows that *P-Cadherin* is expressed in the non-neural ectoderm and in the neural plate border (Fig. 3F-G). *Zeb2* is expressed in the neural ectoderm where *P-Cadherin* expression is absent (Fig. 3F-3H). The neural plate border and the anterior part of the neural plate maintain *P-Cadherin* expression and do not express *Zeb2*. These expression patterns highlight embryonic territories that are becoming different from each other (reviewed by Acloque et al. 2012). The complementary expression of *P-Cadherin* and *Zeb2* is consistent with the idea that *Zeb2* may act as a *P-Cadherin* repressor in the developing neural plate.

**Zeb2 binds to *P-Cadherin* promoter *in vivo***

To assess whether Zeb2 represses *P-Cadherin* expression in the neural plate, we overexpressed Zeb2 in the epiblast of stage HH3 embryos (Fig. 4 A-L). *P-Cadherin* transcripts were downregulated in the electroporated cells (Fig. 4 D-F, n=18/20). Conversely, blocking Zeb2 expression with a morpholino antisense oligonucleotide previously described (Rogers et al., 2013) maintained the expression of *P-Cadherin* in the neural plate at the time when is being downregulated in the control side (Figure 4J-L, n=7/13). ChIP assays to assess whether Zeb2 can bind the E-boxes present in the *P-Cadherin* promoter to repress its activity confirm that as described above for Snail2,

overexpression of a HA-tagged Zeb2 construct (HA-Zeb2) followed by ChIP shows binding to regions B and C of the *P-Cadherin* promoter (Fig. 4M, N). Efficiency and specificity of this experiment were evaluated in a similar manner to that shown in Fig. 2. As for Snail2, these results indicate that Zeb2 can directly bind to and repress transcription of the *P-Cadherin* gene in vivo.

**Snail2 and Zeb2 directly repress *P-Cadherin* expression in the developing chicken embryo**

Once shown that both Zeb2 and Snail2 bind *P-Cadherin in vivo* at regions where *P-Cadherin* expression is downregulated, we examined whether they could directly repress promoter activity by transfecting a *P-Cadherin* promoter reporter construct in the presence or absence of Zeb2 and Snail2 in COS cells (Fig. 4O) and confirmed that both Zeb2 and Snail2 can directly repress *P-Cadherin* transcription. It is worth noting here that Zeb2 does not repress *E-cadherin* expression while Snail2 does (Fig.S4 A-I). This is consistent with the absence of P-cadherin and the presence of E-cadherin protein in the neural tube (Dady et al. 2012) and also with the finding that Zeb2 overexpression is not sufficient to induce EMT in the neural plate cells. Its role in the neural tube is rather modulating the border between neural and non neural ectoderm (Fig. S4 J-R). This indicates that Zeb2 contributes to the definition of neural versus non-neural ectoderm.

Together, our data show that in primitive streak stage chick embryos, P-cadherin is the functional homolog of E-cadherin in mammals and that the sequential activation of different EMT-TFs to repress type I cadherins in the primitive streak and the neural tube is conserved and contributes to the definition of embryonic territories in vertebrates.

## MATERIALS AND METHODS

### Chick embryos and explant cultures

Fertilized hens' eggs were purchased from Granja Gilbert, Tarragona, Spain. The eggs were incubated, opened and the embryos explanted for EC culture as described (Flamme, 1987; Chapman et al. 2001). Embryos were staged according to Eyal-Giladi and Kochav (1976) (EG, in Roman numerals) and Hamburger and Hamilton (1951) (HH, in Arabic numbers).

### Chick Embryos electroporation

Explanted embryos at HH2-HH3 were placed, vitelline membrane and filter paper down, in an electroporation chamber (NEPAGEN) connected to the negative pole of a current pulse generator. A solution containing expression plasmids (2mg/ml in PBS with 0.1% Fast green and 6% sucrose), dsRNA (Pekarik et al. 2003) or morpholinos (MO at 1µM in PBS together with 1µg/µl pCX plasmid, 0.1% Fast green and 6% sucrose) was injected between the vitelline membrane and the epiblast. An anodal electrode was placed over the ventral side of the embryo to cover the injected area. A train of electric pulses (5 pulses, 4 Volts, 50 ms, 0.5 Hz) was applied using an Intracept TSS10 pulse stimulator (Intracel). In all experiments, the non-electroporated right side of the embryo was used as a control.

### DNA constructs

pCX-Snail2, pCX-GFP and pCX-mycSnail2 expression vectors were described previously (Morales et al., 2007, Acloque et al. 2011). Full length Zeb2 was amplified using degenerate primers from sequence alignment of the ATG region of *Xenopus*, human and mouse orthologues 5'-ACCATGAAGCARSNGATCATG-3' and a previously published sequence of the C-terminal region (Sheng et al. 2003). Full length Zeb2 and HA-Zeb2 were sequenced and cloned in pCX at the EcoR1 restriction site.

*P-Cadherin* promoter was amplified by PCR using primers described in Table S1 using KAPA High Fidelity HotStart polymerase and then subcloned at the KpnI restriction site of pGL2-basic (Promega) to produce the p1821-luc plasmid. The whole *P-Cadherin* gene sequence was deposited at Genbank with the Accession Number KY120274.

**Cell transfections and promoter activity assays**

Cell transfections were carried out as described in Acloque et al. (2004) in COS7 cells (free of mycoplasma contamination) and using pRL-TK to normalize for transfection efficiency. 300ng of reporter p1821-luc plamid and either 300ng of empty pCX plasmid or pCX-Snail2 or pCX-Zeb2 were used. Firefly and Renilla luciferase luminescence assays were successively performed using Dual Luciferase Assay (Promega) as described by the manufacturer.

**Whole-mount *in situ* hybridization**

Whole-mount *in situ* hybridization was carried out as described previously (Nieto et al. 1996) but omitting the proteinase K treatment. Digoxigenin-labeled probes were synthesized from the partial or full-length chicken cDNAs of *Snail1*, *Snail2*, *L-CAM*, *N-Cadherin*, *Sox2* (Nieto et al., 1994, Sefton et al. 1998) and from Expressed Sequence Tags (EST; Boardman et al. 2002) for *P-Cadherin* (ChEST913f11) and *Twist1* (ChEST613g12). The Zeb2 probe was generated by RT-PCR and subsequent cloning in pGEMT-easy (Promega). For whole mount fluorescent *in situ* hybridization, embryos were processed as previously described (Acloque et al., 2008). Peroxidase activity was successively detected with the TSA-plus Cy3 and Fluorescein kits (Perkin Elmer). In some cases, embryos were subjected to immunostaining with anti-GFP antibody (A6455, Thermo Scientific, 1:1000). After *in situ* hybridization, embryos were photographed and subsequently embedded in gelatin, sectioned at 40 µm, and photographed using a Leica DMR microscope under Nomarski DIC optics.

**Chromatin immunoprecipitation (ChIP)**

Chick embryos were electroporated either with GFP and control myc-Tag or control HA-Tag, GFP and myc-Snail2 or GFP and HA-Zeb2 expression plasmids. 8 hours after electroporation, GFP positive tissues were dissected from HH5 embryos. ChiP assays were performed as previously described (Acloque et al. 2011). For each assay, we used a pool of 40 embryos (corresponding approximately to $3x10^5$ cells). The following antibodies were used for chromatin immunoprecipitation: anti-myc ChIP grade (ab9132, Abcam), anti-HA ChIP grade (ab9110, Abcam), anti-H3 ChIP grade (ab1791, Abcam) or rabbit IgG control (C15410206, Diagenode) using 1 µg of antibody for each tissue lysate. DNA was amplified by PCR and quantified using H3 samples as a reference.

**PCR and Real Time PCR**

DNA obtained from the ChIP experiments was amplified using primers corresponding to regions B, C and D of the *P-Cadherin* promoter (see Table S1 for sequences). Efficiency of primers designed for real-time PCR amplification of *P-Cadherin, E-Cadherin, N-Cadherin, ACTB* (Voiculescu et al. 2008), *GAPDH* (Voiculescu et al. 2008) *and RS17* (Lavial et al. 2007) was estimated by standard curve production (Table S1). Reverse transcription of total RNA from EGX-XII, HH4, HH9 embryos and chicken embryonic fibroblasts (CEFs) was performed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific), according to the manufacturer's guidelines. Real Time PCRs were performed using SYBR Green mix (Thermo) in a StepOnePlus thermocycler (Thermo). Relative gene expressions were determined using the ΔΔCt method corrected for primer efficiencies with the StepOne Software v2.3 (Thermo), using *RS17* as reference gene (Fig. S3B, C) and HH9 as reference sample (Fig. S3C).

**RNA-seq analysis**

Data from Fresard et al. (2014), (NCBI_SRA accession number: SRP033603) were used for analysis. Transcript sequences from two chicken embryos (d4.5) were aligned using Tophat 2.0.5 (http://ccb.jhu.edu/software/tophat/index.shtml) on the reconstructed *P-* and *E-Cadherin* regions. Data were visualized on IGV2.3. Transcription Start Site was defined by the limit of read alignment at the 5' end of the first exon. Frequency of CASSTG was calculated as follow: random frequency corresponds to one CASSTG site each 1024bp (1/4*4*2*2*4*4). We observed 10 CASSTG in 800bp for B and C regions upstream of the P-cadherin promoters leading to a 12-fold higher frequency than expected at random.

**Nomenclature**

The three chicken type I cadherins were previously named *L-CAM* for *E-Cadherin* (*CDH1*), *N-Cadherin* (*CDH2*) and *K-CAM* or *B-Cadherin* for *P-Cadherin* (*CDH3*). To avoid confusion, we use *E-Cadherin* for *L-CAM* (*CDH1), N-Cadherin* for *CDH2* and *P-Cadherin* for *K-CAM*/*B-cadherin*/*CDH3*.

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## COMPETING INTERESTS

No competing interests declared

## AUTHORS CONTRIBUTIONS

H.A. designed and performed the majority of experiments, analyzed the data and wrote the manuscript; O.H.O. contributed to electroporation experiments; D.A. performed expression studies; C.D.S. interpreted the data, hosted H.A. for the completion of some experiments and contributed to write the manuscript; M.A.N. conceived the project, designed experiments, interpreted the data and wrote the manuscript.

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**Figures**

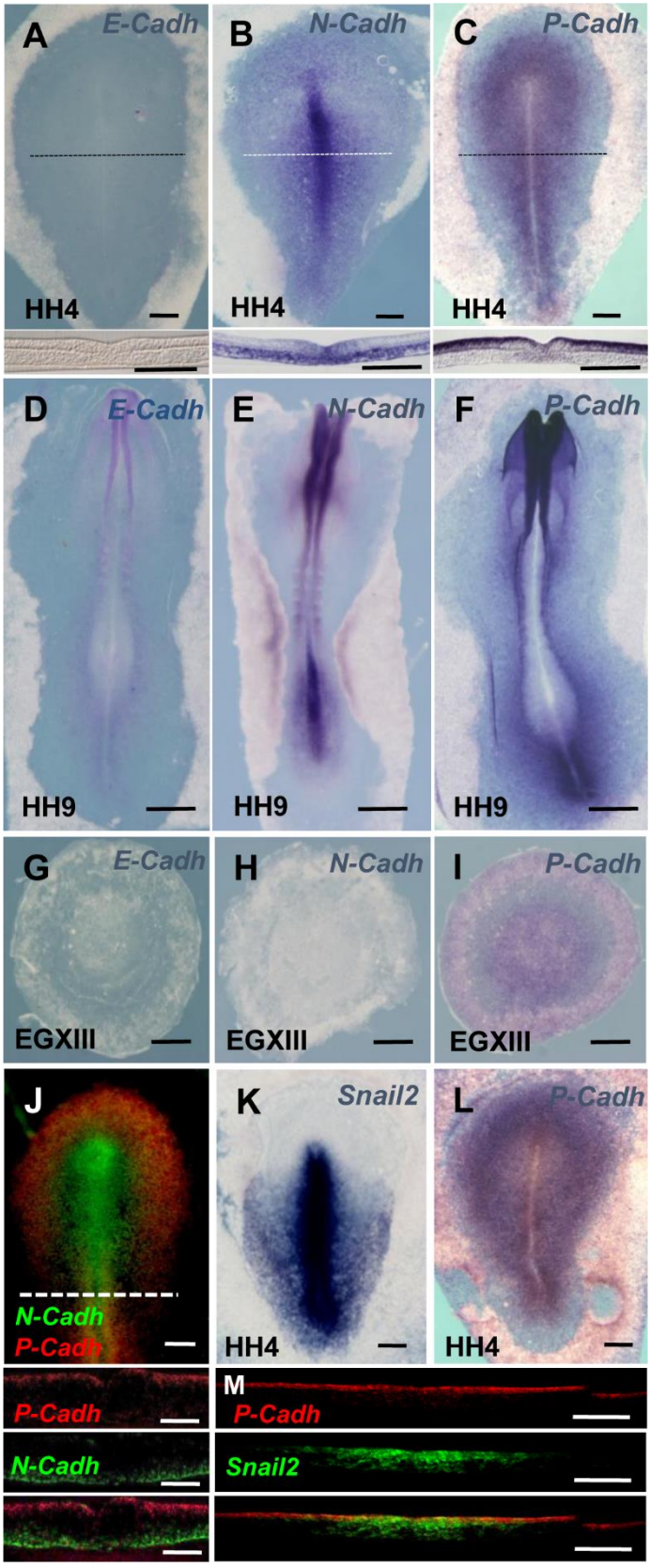


**Figure 1. Expression of Type I cadherins relative to *Snail2* in the early chick embryo.** Whole mount *in situ* hybridization for *E-Cadherin* (L-CAM), *N-Cadherin* and *P-Cadherin* (also called K-CAM or B-cadherin) at stage HH4 (A-C), stage HH9 (D-F) and stage EGXIII (G-I). (J) Double *in situ* hybridizations confirm the complementary expression of *P-* and *N-Cadherin* in primitive streak stage embryos. (K-M) Note the complementary expression between *P-Cadherin* and *Snail2*. The dotted lines indicate the level of the tissue sections. Scale bars represent 500 µm in (A-C, K-L) and 1 mm in (D-F, G-I).

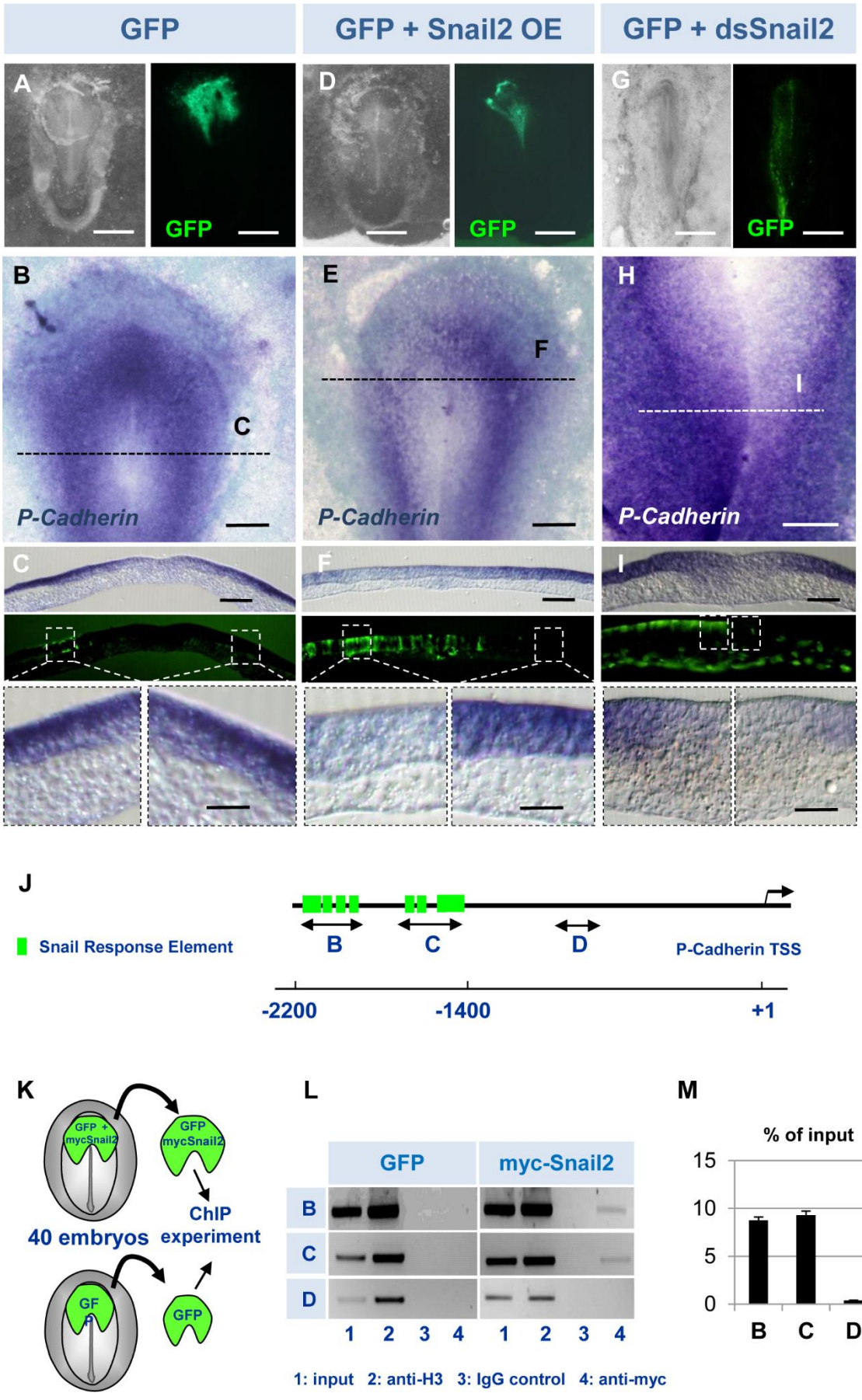


**Figure 2. Snail2 directly represses *P-Cadherin* transcription in the epiblast of primitive streak stage chick embryos.** (A-C) GFP ectopic expression does not affect *P-Cadherin* transcripts in the epiblast of early chick embryos as observed in whole embryos and sections. (D-F) Snail2 overexpression causes downregulation of *P-Cadherin* expression in the epiblast (compare the electroporated with the control side in E and F). (G-I) Knock down of *Snail2* extends *P-Cadherin* expression to the midline, adjacent to the primitive streak (H and sections in I). (J) Specific regions enriched for Snail binding sites (E-boxes with the consensus CASSTG), are located between 2.2kb and 1.4 kb upstream of the *P-Cadherin* TSS (regions B and C). (K) Electroporated prospective neural plate regions ectopically expressing either GFP or GFP together with a myc-tagged Snail2 were dissected and subjected to chromatin immunoprecipitation (ChIP) assays. (L, M) DNAs from the chromatin enriched fraction for the regions described in (J) were amplified by PCR. Amplification from input DNA is shown in lane 1 and positive and negative controls using either a pan-H3 antibody or an IgG control are shown in lanes 2 and 3. When myc-Snail2 was expressed in the epiblast, regions B and C were specifically enriched in the immunoprecipitated fractions, detected with an anti-myc antibody (compare the GFP control conditions shown in lane 4). The quantification of enrichment is shown in M. Bars represent mean ± s.d of three independent experiments. Scale bars represent 1 mm in A, D, G, 400µm in B-C, E-F, H-I and 150µm in the magnified panels of the sections.

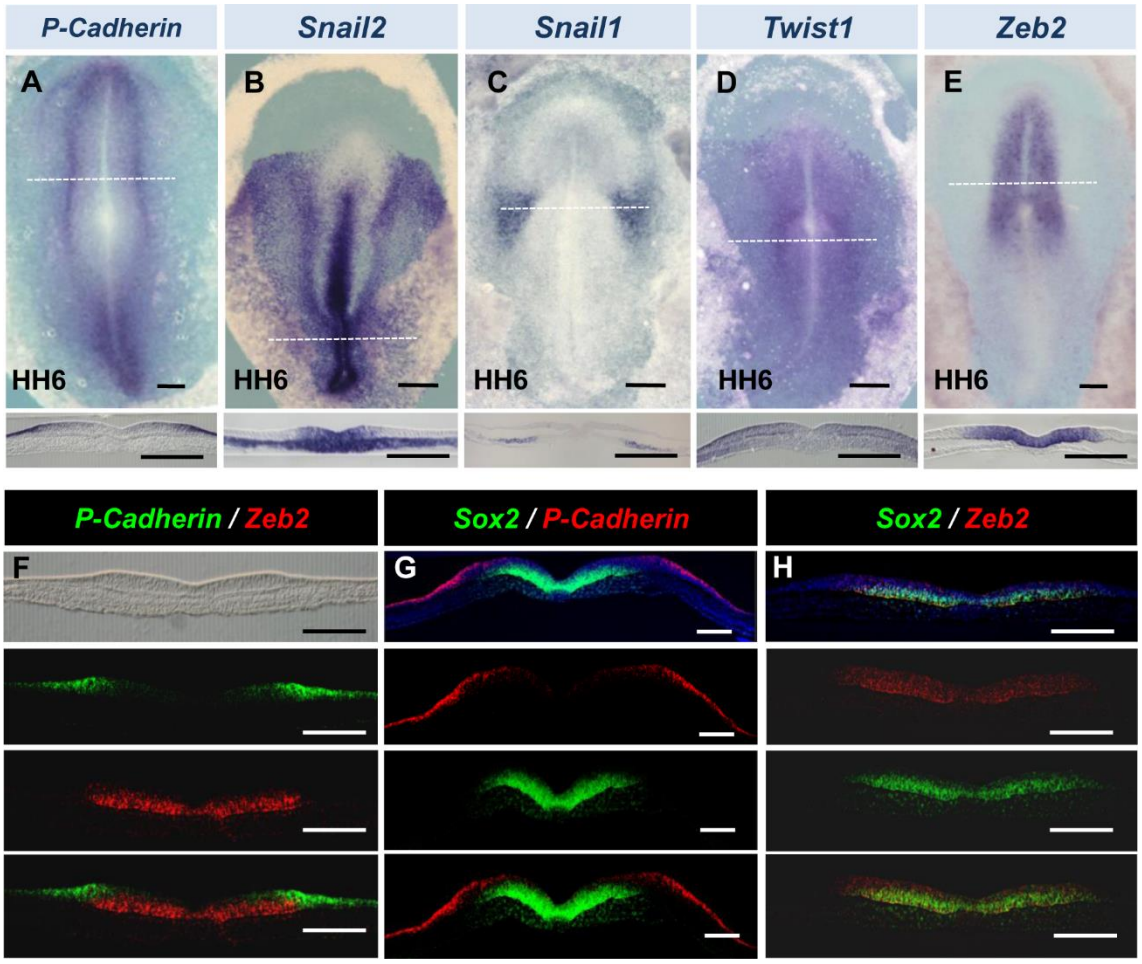



**Figure 3. Expression of EMT inducers relative to *P-Cadherin* at early neurulation stages**

(A) *P-Cadherin* is downregulated in the early neural plate. (B-C) *Snail* genes are mostly expressed in the mesoderm. (D) Twist is weakly expressed in the lateral mesoderm and the ectoderm. (E) *Zeb2* is strongly expressed in the early neural plate, at sites where *P-Cadherin* is downregulated. (F-H) Expression of *Zeb2* and *P-Cadherin* relative to *Sox2*. P-cadherin is expressed in the non-neural ectoderm and at the border of the neural plate, while *Zeb2* transcripts are specifically detected in a S*ox2* positive region of the neural ectoderm devoid of *P-Cadherin* expression. The dotted lines indicate the level of the tissue sections. Scale bars represent 500µm in (A-E), 300µm in (F-H).

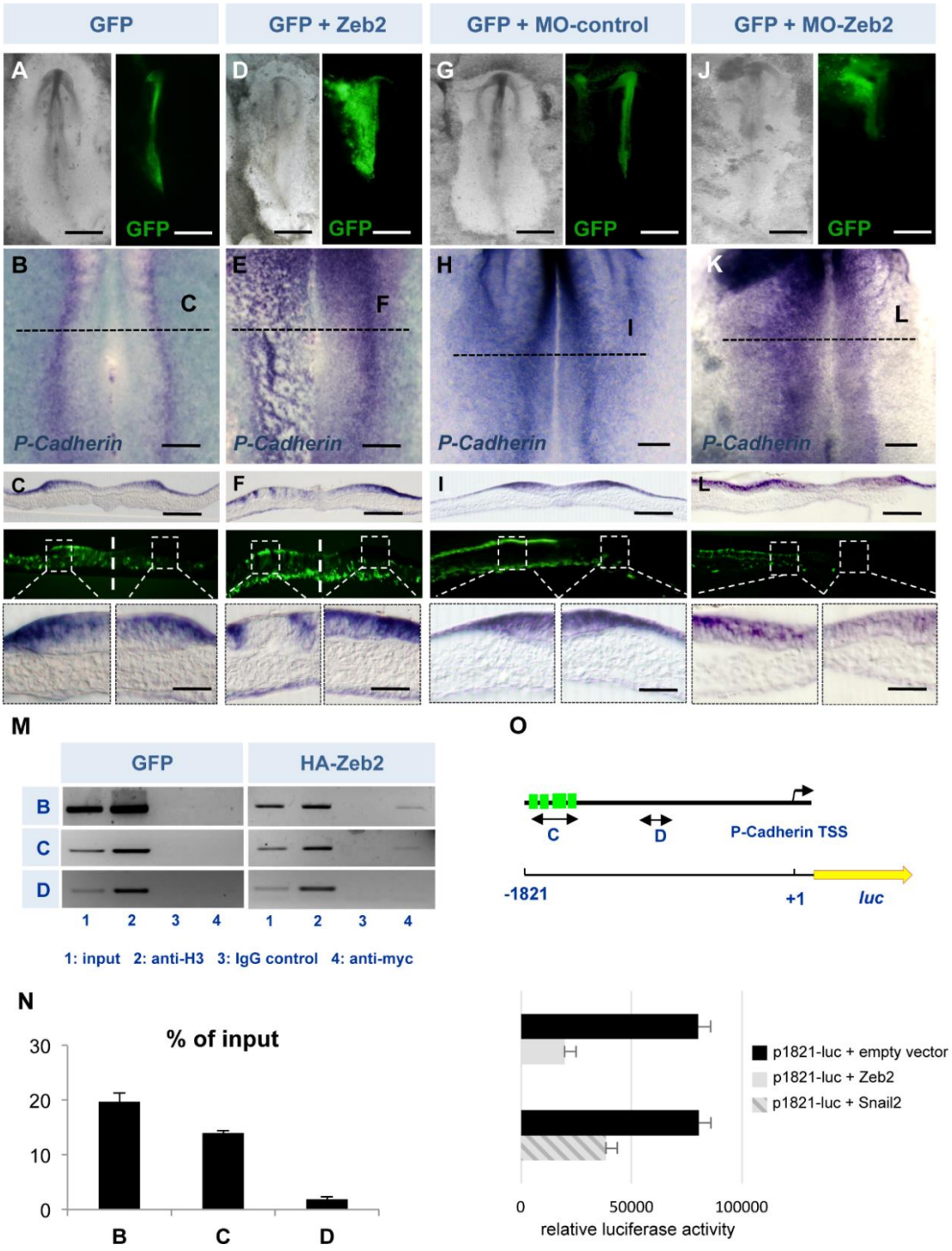



**Figure 4. Zeb2 directly represses *P-Cadherin* transcription in the neural plate.**

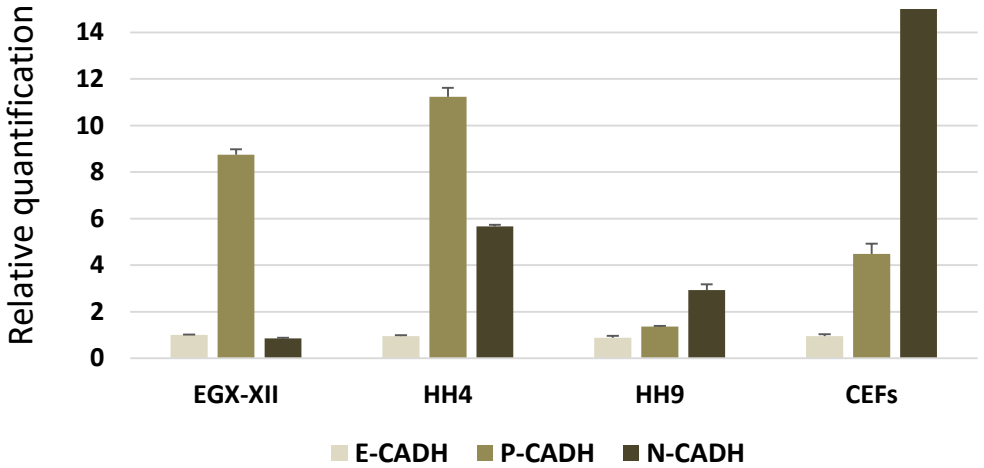
(A-C) GFP electroporation does not affect *P-Cadherin* expression in the neural plate of HH7 embryos. (D-F) Zeb2 overexpression represses *P-Cadherin* expression in the neural ectoderm (compare the electroporated versus the control side). (G-I) Control morpholino (MO-control) electroporation does not affect *P-Cadherin* expression in the neural plate of HH7 embryos. (J-L) Zeb2 downregulation after electroporation of a morpholino directed

against the ATG (MO-Zeb2) increases *P-Cadherin* expression and extends it to the midline of the embryo. (M) Zeb2 binds to *P-Cadherin* promoter *in vivo.* Chromatin immunoprecipitation (ChIP) from electroporated neural plates overexpressing either GFP alone or together with a HA-tagged Zeb2. DNA from the chromatin enriched fraction was amplified by PCR. Amplification from input DNA is shown in line 1, and positive and negative controls using either a panH3 antibody or an IgG control are shown in lines 2 and 3, respectively. Regions B and C are specifically enriched using an antibody directed against HA when HA-Zeb2 was electroporated in the epiblast (compare GFP with HA-Zeb2 conditions in line 4). (N) Quantification of the enrichment shown in (M). Regions B, C, and D are described in Fig. 2. Bars represent mean ± s.d. of three independent experiments. (O) Diagram of the reporter construct (p1821-luc) used to quantify *P-Cadherin* promoter activity. COS7 cells were transfected with p1821-luc together with an empty expression vector (black bars), with a Zeb2 expression vector (grey bar) or with a Snail2 expression vector (striped grey bar). Bars represent mean ± s.d. of two independent experiments. Scale bars represent 1mm in (A, D, G, J), 500µm in (B-C, E-F, H-I, K-L), 150µm in the higher magnification panels of the sections.

**A**

| | mean of observed Ct | | | | | |
|---|---|---|---|---|---|---|
| | RS17 | ACTB | GAPDH | E-CADH | P-CADH | N-CADH |
| **EGX-XII** | 17.9 | 17.2 | 16.5 | 23 | 19.9 | 23.3 |
| **HH4** | 19.4 | 18.8 | 18.9 | 25.4 | 21.9 | 22.9 |
| **HH9** | 16.8 | 15.9 | 15.5 | 21.6 | 21.1 | 20 |
| **CEFs** | 19.3 | 18.1 | 18.3 | 30.3 | 28.2 | 21.3 |

**B**




**C**

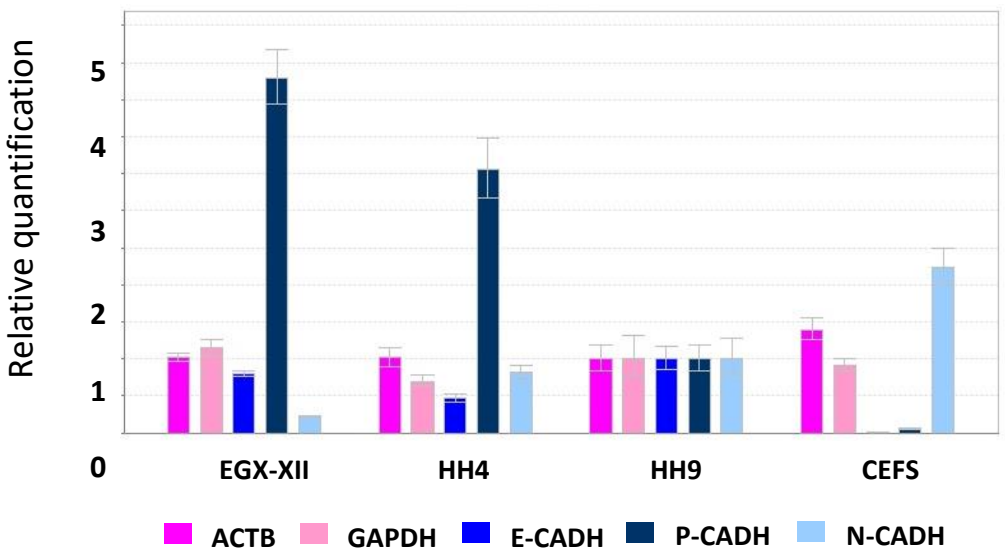



**Figure S1: Relative abundance of *E-Cadherin*, *P-Cadherin* and *N-Cadherin* transcripts in chicken early embryos determined by real-time PCR.**
(A) Mean of observed Ct (threshold cycle) for the genes analysed at different embryonic stages. (B**)** Relative abundance of *P-Cadherin* and *N-Cadherin* transcripts relative to those of *E-Cadherin* after normalization with *RS17* at the different embryonic stages. (C) Relative abundance of *ACTB*, *GAPDH*, *E-Cadherin*, *P-Cadherin* and *N-Cadherin* at the different embryonic stages relative to those at HH9 and after normalization with *RS17* (ΔΔCt method). Bars represent mean ± s.d of three independent experiments,

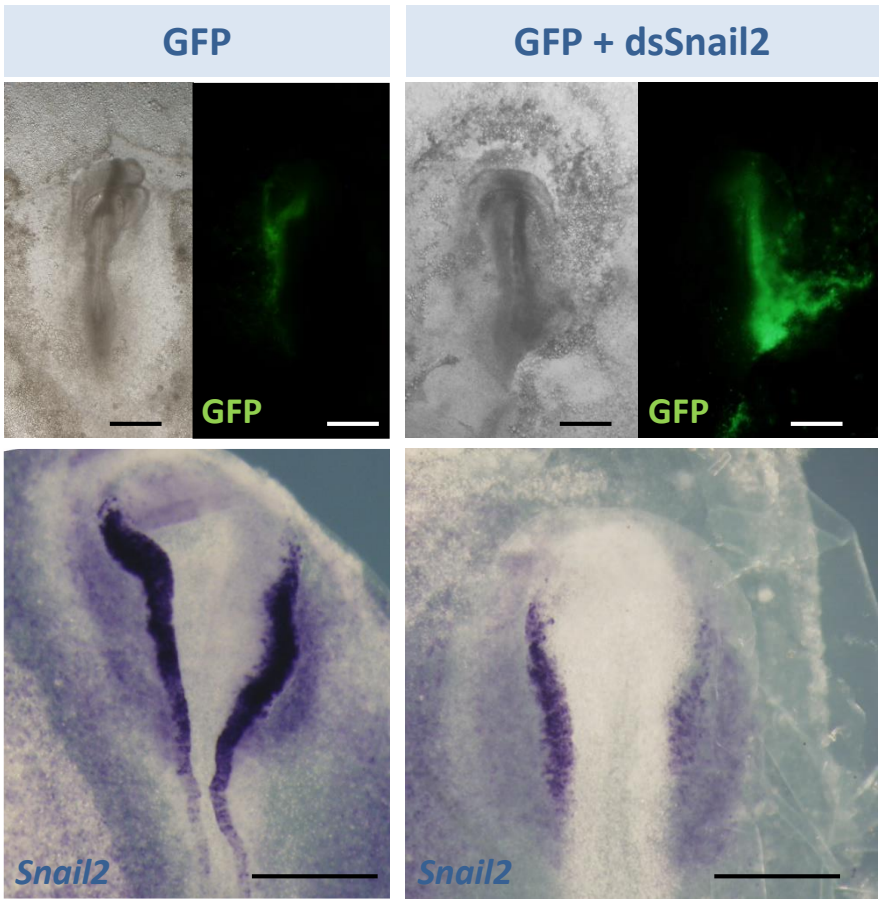
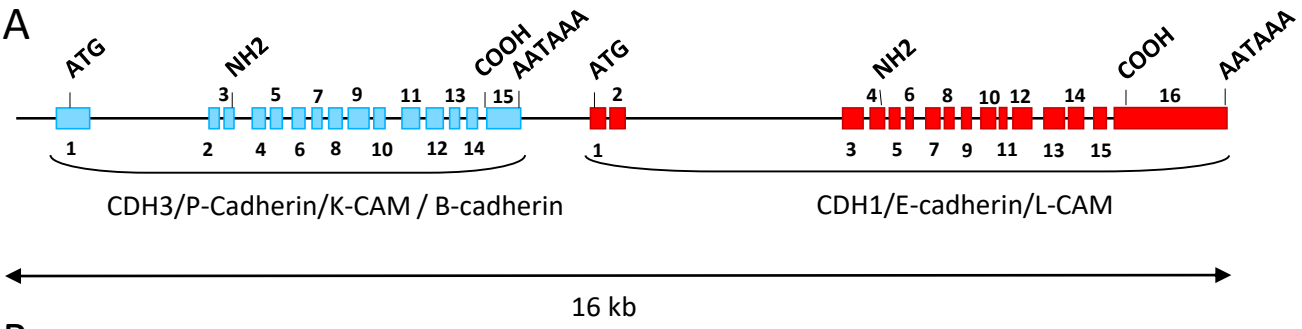
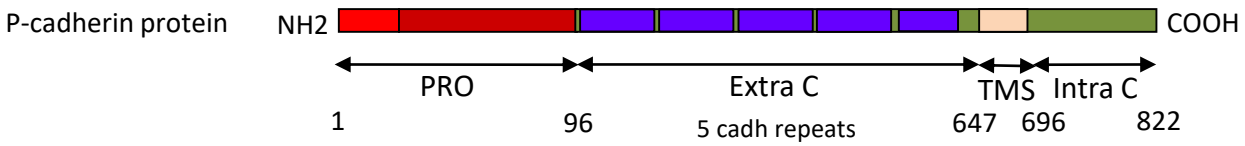



**Figure S2. Downregulation of *SNAIL2* mRNA by ds*SNAI2***
The electroporated region (right side of the embryo) shows a slight decrease of *Snail2* expression in 100% of the electroporated embryos (compare the left and right sides, n=3). GFP electroporation does not affect *Snail2* expression in the neural fold of HH8 embryos. Scale bars represent 1mm.

A



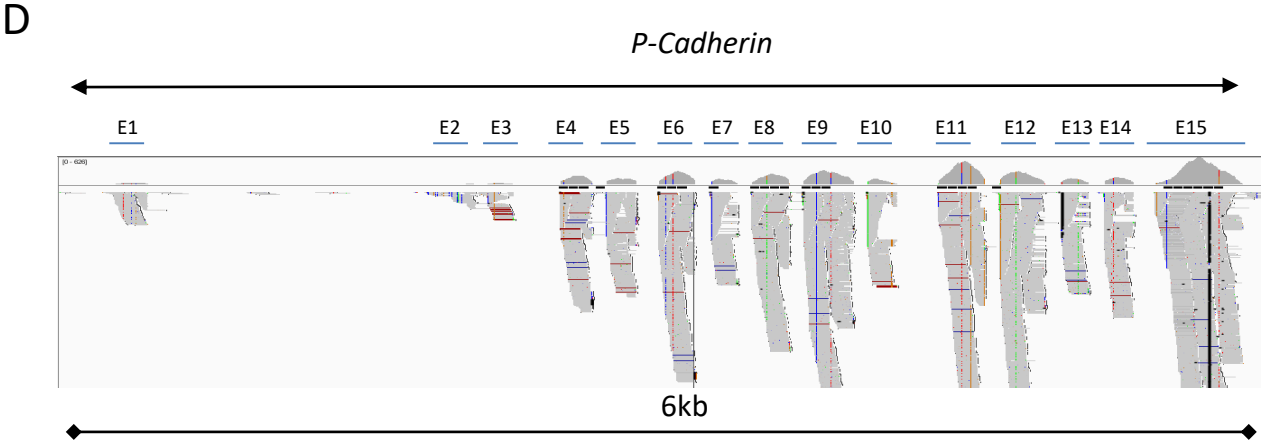

B




C

| | Intra C Chick P-Cadherin | TMS Chick P-Cadherin | Extra C Chick P-Cadherin |
|---|---|---|---|
| | | % of identity | |
| **Chick P-Cadherin** | 100 | 100 | 100 |
| **Chick E-Cadherin** | 100 | 68 | 59 |
| **Human P-Cadherin** | 83 | 76 | 63 |
| **Human E-Cadherin** | 89 | 81 | 60 |

D




E

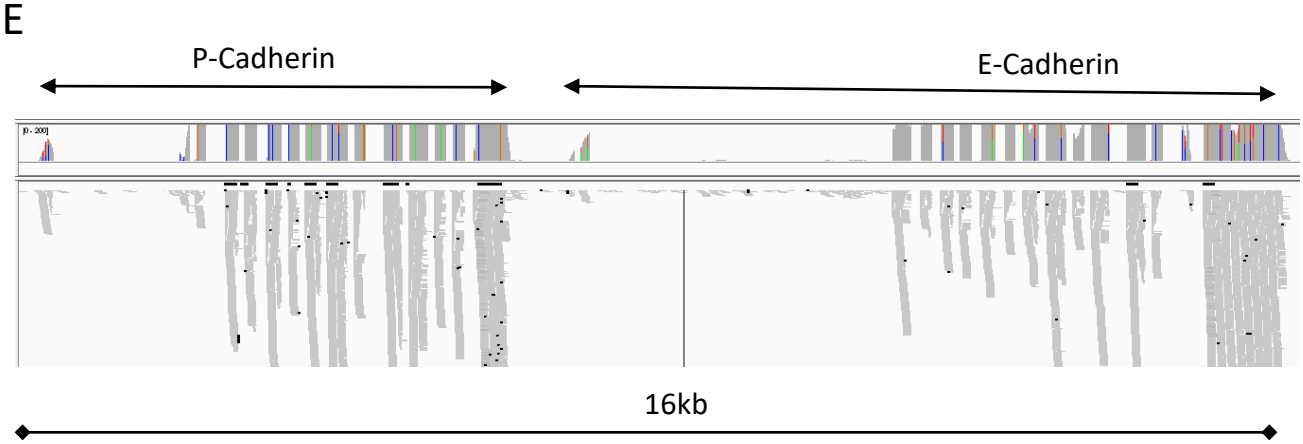
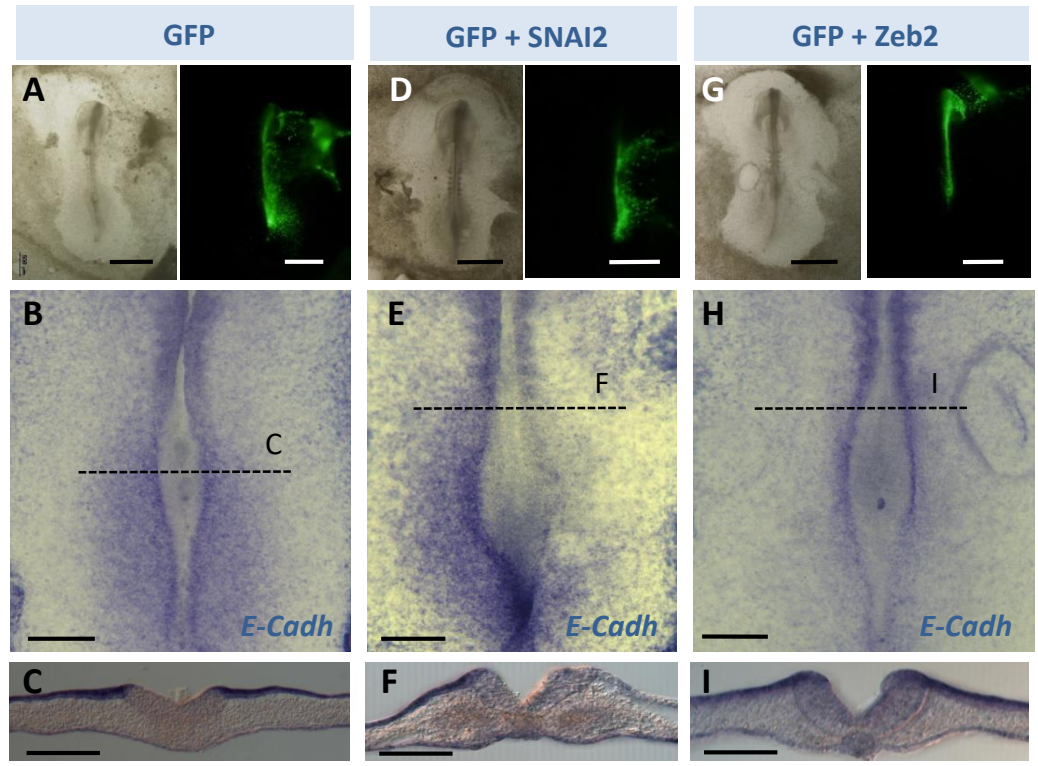


**Figure S3: The *P-Cadherin* (*K-CAM/B-Cadherin*) locus**

(A) The *P-Cadherin* gene is 5695bp long, composed of 15 exons (E) and encoding a 822 aminoacids protein. It is located at the vicinity of the *E-Cadherin* gene and was previously described as a tandem duplication of the L-CAM locus (Sorkin et al. 1991). (B) The unprocessed protein contains a PRO domain (cleaved at amino acid 96 to produce the functional type I P-cadherin), an Extracellular domain (Extra-C containing five cadherin repeats), a Transmembrane Segment (TMS) and a cytoplasmic domain (Intra-C) (Brasch et al. 2012). (C) Sequence homologies between human and chicken *E-Cadherin* and *P-Cadherin* for the cytoplasmic, Intra-C, extra-C and TMS domains. The conserved sequence of the cytoplasmic domain explains why both chicken P- and E-Cadherin are recognized by antibodies designed against the C-terminal part of mammalian E-Cadherin. (D-E) *P-Cadherin* and *E-Cadherin* genomic organization was validated by analysis of a RNA-seq dataset from HH24 chicken embryos. Reads from RNA-seq datasets from whole chick embryos were aligned to the genomic sequence covering chicken *P-* and *E-Cadherin*. Reads at the different exons confirm the TSS and exon structure of the two genes.


GFP
GFP + SNAI2
GFP + Zeb2
A
D
G
B
C
E
F
H
I
E-Cadh
E-Cadh
E-Cadh
C
F
I

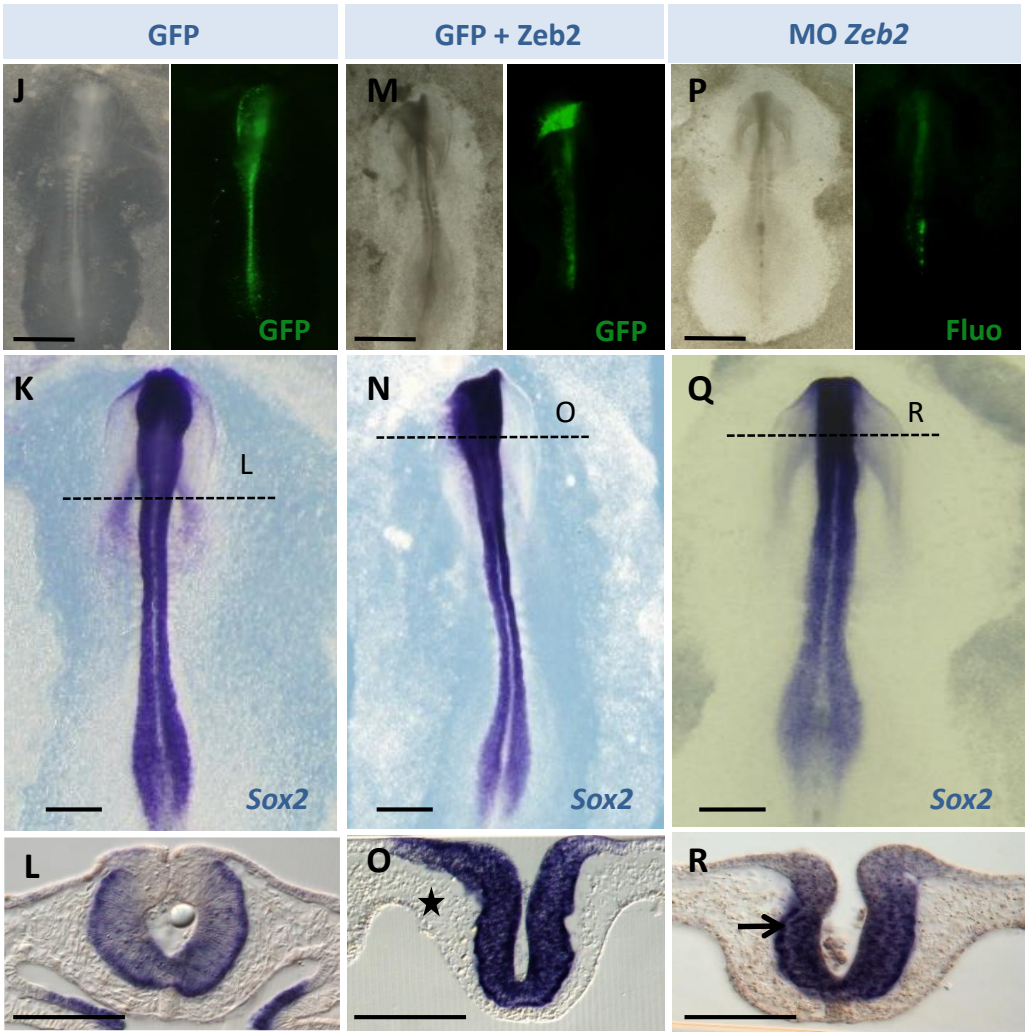

GFP
GFP + Zeb2
MO Zeb2
J
M
P
GFP
GFP
Fluo
K
L
N
O
Q
R
Sox2
Sox2
Sox2
L
O
R

**Figure S4. Zeb2 contributes to the definition of the neural versus non-neural territorries.**
(A-C) GFP ectopic expression does not affect *E-Cadherin* transcripts in the non-neural ectoderm of HH8 chick embryos as observed in whole embryos and sections. (D-F) Snail2 overexpression causes downregulation of *E-Cadherin* transcripts in the non-neural ectoderm (compare the electroporated with the control side in E and F, n=3/4). (G-I) Zeb2 overexpression does not affect *E-Cadherin* expression (n=5/5). (J-L) GFP ectopic expression does not affect *Sox2* transcripts in the neural tube as observed in whole embryos and sections (n=5/5). (M-O) Zeb2 overexpression expands *Sox2* expression (n=6/8) while Zeb2 knock-down (P-R) reduces *Sox2* expression in the dorsal neural tube (arrow and compare the electroporated with the control side in O and R, n=3/4).
Scale bars represent 1mm in (A, D, G, J, M, P) and 500µm in (B-C, E-F, H-I, K-L, N-O, Q-R),

**Table S1**
Primers used in this study for QPCR and PCR analysis

| Gene | | Sequence 5'-3' | Position | Efficiency from standard curves |
|---|---|---|---|---|
| **RS17** | F | ACACCCGTCTGGGCAACGACT | Exon 1 | 102% ; $R^2$ 0.996 |
| | R | CCCGCTGGATGCGCTTCATCA | Exon 2 | |
| **E-Cadherin** | F1 | TGTACGAAGGTGTGGTGGAA | Exon 9 | 95%; $R^2$=0.998 |
| | R1 | AGCGGCTCTTGGTCTCATAA | Exon 10 | |
| **E-Cadherin** | F2 | CCAGGATGTGAATGACAACG | Exon 12 | 90%; $R^2$=0.997 |
| | R2 | CATCTTGGCCCCTTATCTCA | Exon 12/Exon13 | |
| **N-Cadherin** | F1 | TCTTGATGAAAGGCCAATCC | Exon 15 | 87%; $R^2$=0.997 |
| | R1 | AGCCGCTTCCTTCATAGTCA | Exon 16 | |
| **N-cadherin** | F2 | TCCTGACCGATCCAAATAGC | Exon 9 | 100%; $R^2$=0.999 |
| | R2 | ACGTACAAGCTTGGGGTTTG | Exon 10 | |
| **P-Cadherin** | F1 | AGGGCATCTTCACCATTGAG | Exon 4 | 94%; $R^2$= 0.995 |
| | R1 | GAACTGGGGCTTGTTGTCAT | Exon 5 | |
| **P-Cadherin** | F2 | TACAACGGTGTCATCGCCTA | Exon 6 | 94%; $R^2$= 0.998 |
| | R2 | CTGCATGGTCAGCGTGTACT | Exon 7 | |
| **ACTB** | F | GCCCCTCCCTCCCCCACATA | Exon 5 | 85% ; $R^2$ 0.995 |
| | R | GGAGCGAACGCCCCCAAAGT | Exon 5 | |
| **GAPDH** | F | GTGGGGGAGACAGAAGGGAAC | Exon 8 | 90% ; $R^2$ 0.997 |
| | R | AGAGGTGCTGCCCAGAACATC | Exon12 | |
| **P-Cadh B region** | F | AGTACCATGCTGTGCCTCCT | | |
| | R | CAACAGCTGGCCTCAACAG | | |
| **P-Cadh C region** | F | CTGGAAGCAGCCATGTCC | | |
| | R | GACGTCCCACCCCATAGAG | | |
| **P-Cadh D region** | F | ATGGTGGACCTGAGCCCGG | | |
| | R | GTCTGTCCAACACCGCCACG | | |
| **Zeb2 probe** | F | GTTTAGTGCAACTAGTCCTT | | |
| | R | TATTACATGCCATCTTCC | | |
| **P-Cadh prom** | F | NNGGTACC AAGCAGCCATGTCCCCATC | | |
| | R | CGAGCAGCAGGAGAAGGAA | | |